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review

Phosphoinositide-metabolizing enzymes at the interface between membrane traffic and cell signalling

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Phosphoinositides (PIs) have long been known to have important roles in cell signalling. During the past decade, it has become clear that these lipids also act as constitutive signals that aid in defining organelle identity, and are short-lived recruiters and regulators of cytoskeletal and membrane dynamics. Recent studies have provided important clues as to how regulated activation of PI-metabolizing enzymes and recruitment of their binding proteins might cooperate in targeting distinct pools of PIs to different cell physiological functions.

Keywords: cell signalling; membrane traffic; phosphoinositide kinases; phosphoinositide phosphatases; phosphoinositides

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Introduction

Dynamic membrane flux is intimately linked with intracellular signalling events that are initiated at the plasma membrane or within intracellular organelles (Polo & Di Fiore, 2006). During the past decade, it has become clear that lipids have essential roles in the spatio-temporal regulation of membrane trafficking, in addition to their established roles in cell motility, adhesion and signal transduction (Berridge & Irvine, 1989; Martin, 1998). Most prominent among these lipids are the phosphoinositides (PIs), which are short-lived phosphorylated derivatives of phosphatidylinositol (De Matteis & Godi, 2004; Di Paolo & De Camilli, 2006). Accumulating evidence indicates that different organelles contain distinct sets of accessible PIs (Fig 1). For example, the plasma membrane contains much more free phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) than phosphatidylinositol 4-phosphate (PI(4)P), whereas in Golgi membranes this ratio is reversed (De Matteis & Godi, 2004). Phosphatidylinositol 3-phosphate (PI(3)P) and its derivative phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) confer identity to endosomal membranes (Lindmo & Stenmark, 2006). Furthermore, PIs are presumed to be concentrated within spatially restricted subdomains, so that defined pools of PIs can be targeted to specific cellular activities. PI distribution is governed by the finely tuned balance of the enzymatic activities of lipid kinases and phosphatases that are controlled by intracellular signalling cascades (Table 1).

How precisely specific PIs are targeted to a distinct cell physiological process, perhaps even within the same membrane compartment, remains an unresolved, but important, question. In this review, we summarize potential mechanisms underlying the localized generation of distinct PIs that are targeted to specific cellular functions (Fig 2). Furthermore, we give examples of how signalling processes can provide an additional layer of regulation to modulate local PI pools.

Locally restricted $PI(4,5)P_2$ synthesis

An obvious mechanism for generating and maintaining distinct subcellular pools of PIs is exemplified by the spatial and temporal control of $PI(4,5)P_2$ synthesis (De Matteis & Godi, 2004; Di Paolo & De Camilli, 2006). $PI(4,5)P_2$ synthesis at the plasma membrane is mediated predominantly by type I phosphatidylinositol-4-phosphate 5-kinases (PIP(5)Ks), which phosphorylate PI(4)P at the 5′ position of its inositol group. $PI(4,5)P_2$ is an essential cofactor for various cellular processes, including clathrin-dependent and clathrin-independent modes of endocytosis, calcium-dependent secretion of hormones and neurotransmitters, rearrangements of the actin cytoskeleton, cell migration and integrin-mediated cell-matrix adhesion (De Matteis & Godi, 2004; Di Paolo & De Camilli, 2006; Ling *et al*, 2006).

Precisely how $PI(4,5)P_2$ regulates such a broad spectrum of cellular functions is unclear. However, several distinct recruiters of type I PIP(5)Ks have been identified that target kinase activity to distinct sites within the plasma membrane. Among these are the small G proteins Rho, Rac and cell-division cycle 42 (Cdc42), which regulate $PI(4,5)P_2$-mediated rearrangements of the actin cytoskeleton (Santarius *et al*, 2006). Similarly, the small GTPase ADP-ribosylation factor 6 (ARF6) has been implicated in $PI(4,5)P_2$-dependent trafficking processes at the plasma membrane (Donaldson, 2005). ARF6-GTP associates directly with type I PIP(5)Ks (Di Paolo & De Camilli, 2006; Santarius *et al*, 2006), and is coupled to distinct cell signalling events through different guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs).

PI kinase activation is often influenced by several cooperating factors that create a positive feed-forward loop. For example, ARF6 activates isoforms of phospholipase D (PLD). The product of PLD action—phosphatidic acid—is an important cofactor for the stimulation of type I PIP(5)Ks. In addition, $PI(4,5)P_2$ activates PLD.

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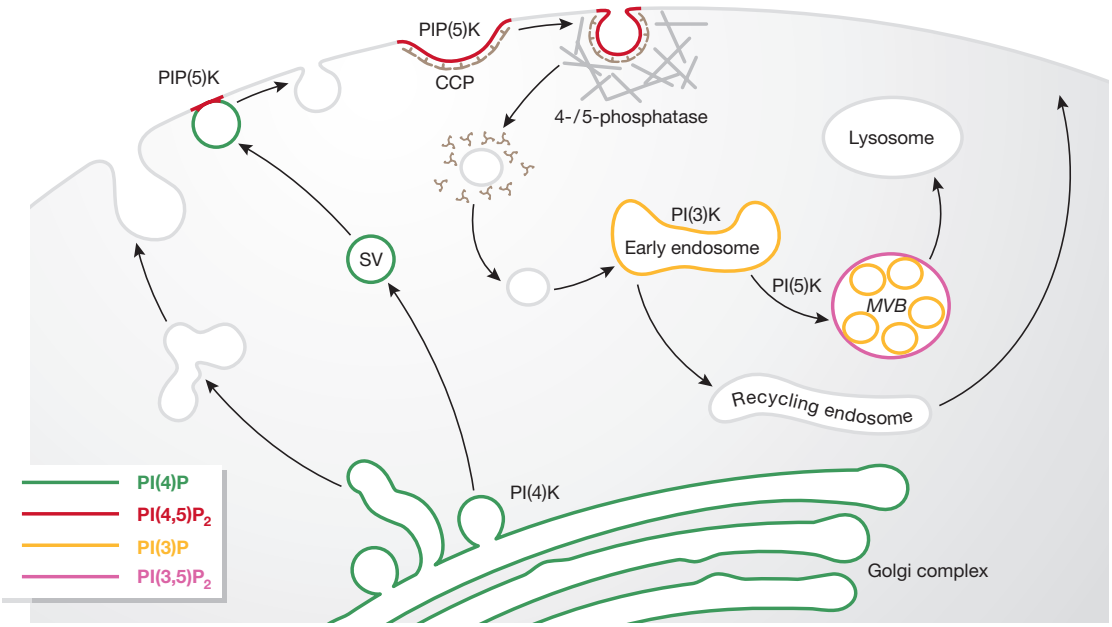

Fig 1 | Phosphoinositides and their metabolizing enzymes in exo-endocytic membrane traffic. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and the corresponding metabolizing enzymes are required for fusion of secretory vesicles (SVs), formation of endocytic clathrin-coated pits (CCPs) and changes in actin dynamics. Phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) delineate sorting stations for endocytic cargo through multivesicular bodies (MVBs) en route to the lysosome. Phosphatidylinositol 4-phosphate (PI(4)P) confers identity to the Golgi complex, and is required for trafficking of secretory cargo and perhaps lysosomal enzymes (not shown). PIs, phosphoinositides; PI(3)K, phosphatidylinositol 3-kinase; PI(4)K, phosphatidylinositol 4-kinase; PI(5)K, phosphatidylinositol 5-kinase; PIP(5)K, phosphatidylinositol phosphate 5-kinase.

Therefore, ARF6 has synergistic effects on the activities of PIP(5)K and PLD (Jovanovic *et al*, 2006). A synergistic role for PLD in regulating $PI(4,5)P_2$-dependent processes is supported by the observation that the PHOX homology domain of PLD exhibits GAP activity towards the large endocytic GTPase dynamin during stimulation-induced endocytosis of epidermal growth-factor receptors (EGFRs) and insulin receptors (Lee *et al*, 2006). Localized $PI(4,5)P_2$ synthesis is, indeed, required for clathrin-mediated endocytosis of different receptors. PIP(5)Ks—through their kinase core domains—bind to and are activated by the clathrin adaptor protein complex 2 (AP-2) after its association with cargo membrane receptors (Krauss *et al*, 2006). The coincidence detection of membrane cargo and $PI(4,5)P_2$ by AP-2 paired with AP-2-cargo complex-mediated activation of PIP(5)K could therefore result in the generation of a local pool of $PI(4,5)P_2$ specifically dedicated to clathrin/AP-2-dependent endocytosis.

Furthermore, spatially restricted $PI(4,5)P_2$ formation can be accomplished by select activation of distinct PI kinase isozymes. In neurons, the p90 splice isoform of PIP(5)K type Iγ (Di Paolo *et al*, 2004) is the major PI(4,5)P-generating enzyme and might be concentrated at nerve terminals through the interaction of a splice-isoform-specific peptide with talin, which is a scaffolding protein that connects integrins and the actin cytoskeleton. Talin is also found at focal adhesion sites in non-neuronal cells, where it enhances the local $PI(4,5)P_2$ concentration by recruiting and activating PIP(5)K Iγ-p90 (Di Paolo & De Camilli, 2006; Ling *et al*, 2006). The presence of a tyrosine-based cargo-like internalization motif within the tail domain of the p90 splice-variant of type Iγ PIP(5)K also aids in stabilizing its interaction with AP-2 at focal adhesions (Fig 3, top; Bairstow *et al*, 2006) and in synapses.

Regulated synthesis of intracellular PI pools

Small GTPases also have important roles in generating and maintaining pools of PI(4)P in Golgi membranes (De Matteis & Godi, 2004). Activated ARF1 recruits type III phosphatidylinositol 4-kinase (PI(4)K) to the trans-Golgi network (TGN), and concomitantly regulates membrane binding of the PI(4)P-binding adaptor protein AP-1 (Wang *et al*, 2003), and of Golgi-associated γ-ear-containing ARF-binding proteins (GGAs) as well as clathrin. How precisely PI(4)K isozymes (Balla & Balla, 2006) are controlled and how this relates to the formation of membrane trafficking carriers remain largely open questions. On the basis of what we know about $PI(4,5)P_2$ synthesis at the plasma membrane, it could be envisioned that a network of regulatory interactions between PI(4)K isozymes, small GTPases and trafficking adaptors or scaffolding proteins aids the formation of distinct pools of PI(4)P destined for different trafficking pathways within the Golgi–endosomal system. In support of this, PI(4)K type IIβ contains a peptide resembling an acidic cluster di-leucine-type sorting motif that could interact with AP-1 or GGA adaptors, in a manner similar to the mechanism described above for the regulation of PIP(5)K type I by AP-2 (Fig 3, top).

Endosomal PI(3)P synthesis is initiated soon after or concomitantly with clathrin-mediated endocytosis. Phosphatidylinositol 3-kinase (PI(3)K)-C2α is a component of the clathrin-transport machinery, and has been shown to be recruited and stimulated by

Table 1 | Phosphoinositide-metabolizing enzymes

Enzymatic activity	Isoform/gene	Predominant substrate	Localization	Disease
PI kinases				
PI(3)K	P110α–δ	PI(4,5)P_2	PM, N	Cancer (P110α)
	PI(3)K-C2α	PI (others?)	PM, TGN/E	–
	PI(3)K-C2β	PI (others?)	PM	–
	PI(3)K-C2γ	PI (others?)	GC	–
	Vps34	PI	GC, E	Bipolar disorder
PI(4)K	PI(4)K IIα	PI	GC, TGN/E; PM (some)	Bipolar disorder
	PI(4)K IIβ	PI	GC, TGN/E; PM (some)	–
	PI(4)K IIIα	PI	ER, GC	–
	PI(4)K IIIβ	PI	N, GC	–
PI(5)K	PIK-fyve	PI(3)P	LE	Francois–Neetens fleck cornea dystrophy
PIP(4)K	PIP(4)Kα,β,γ	PI(5)P	PM (α,β); ER (γ)	–
PIP(5)K	PIP(5)Kα,β,γ	PI(4)P	PM	–
PI phosphatases				
3-phosphatases	PTEN1,2	PI(3,4)P_2, PI(3,4,5)P_3	PM, GC, N	Cancer; Cowden disease
	MTM1	PI(3)P, PI(3,5)P_2	PM	Myopathy
	MTMR1–8	PI(3)P, PI(3,5)P_2	PM; some unknown	Charcot-Marie-Tooth type 4B1 (MTMR2)
4-phosphatases	Sac1-3	PI(4)P	ER, GC	–
	Synaptojanin1,2	PI(4)P?	–	–
	Type I, II	PI(3, 4)P_2	Unknown	–
5-phosphatases	Synaptojanin1,2	PI(4,5)P_2 and others	SV, PM, Mi	Bipolar disorder, Down syndrome
	OCRL	PI(4,5)P_2	TGN/E, Ly	Oculocerebrorenal syndrome of Lowe
	SKIP	PI(4,5)P_2	ER, PM	–
	PIPP	PI(4,5)P_2	PM	–
	SHIP1	PI(3,4,5)P_3	N, PM	Myeloid leukaemia; Paget's disease?
	SHIP2	PI(3,4,5)P_3	N, PM	Type II diabetes
	72kD-5-phosphatase	PI(3,4,5)P_3	GC	–
	5-phosphatase II	PI(3,4,5)P_3	Unknown	–
	5-phosphatase IV	PI(3,4,5)P	Unknown	–

E, endosome; ER, endoplasmic reticulum; GC, Golgi complex; hSac1-3, human homologues of yeast suppressor of actin 1 (Sac1); Vps34, human homologue of yeast Vps34p; LE, late endosome; Ly, lysosome; Mi, mitochondria; MTM1, myotubularin 1; MTMR1–8, MTM1-related proteins; N, nucleus; OCRL, oculocerebrorenal syndrome of Lowe; PI, phosphoinositide; PI(3)K, phosphatidylinositol 3-kinase; PI(4)K, phosphatidylinositol 4-kinase; PI(5)K, phosphatidylinositol 5-kinase; PIP(4)K, phosphatidylinositol phosphate 4-kinase; PIP(5)K, phosphatidylinositol phosphate 5-kinase; PIPP, proline-rich inositol polyphosphate 5-phosphatase; PM, plasma membrane; PTEN, phosphatase and tensin homologue; SHIP1-2, SH2-containing inositol phosphatases 1 and 2; SKIP, sphingosine kinase type 1-interacting protein; SV, secretory vesicle; TGN, trans-Golgi network.

clathrin residing at the plasma membrane or the TGN/endosomal boundary (Gaidarov *et al*, 2001). Another crucial regulator of the endosomal PI(3)P pool is the small GTPase Rab5, which interacts with PI(3)K type I and the human homologue of yeast Vps34p (Vps34). Several endosomal regulators of Rab5 function have been identified, including Rab-GDP-dissociation inhibitor, RabGAP-5, and members of the Rin family of exchange factors, such as Rabex-5 (Lindmo & Stenmark, 2006). In yeast, endosomal Vps34-mediated PI(3)P generation is triggered by mating pheromone-induced Gα activation downstream of G-protein-coupled receptor signalling (Slessareva *et al*, 2006).

Keeping PIs in place: the role of PI-binding proteins

Numerous protein domains have been identified that promote association with distinct PI headgroups (Takenawa & Itoh, 2006), including E/ANTH, PH, PX, PHOX, FYVE, FERM, BAR and C2 domains (Fig 2B). They are frequently combined with additional modules that are involved in the formation of complex protein–protein and protein–lipid interaction networks. The mesh of endocytic proteins provides one of the best examples for such a network: adaptors that recognize cargo proteins at the plasma membrane—that is, AP-2, epsin, disabled 2 (Dab2), autosomal recessive hypercholesterolaemia protein (ARH), huntingtin-interacting protein 1 (HIP1) and the HIP1-related protein—frequently bind to PI(4,5)P_2, as well as to other endocytic proteins, thereby aiding their incorporation into coated pits (Takenawa & Itoh, 2006). Similarly, binding sites for PI(4)P have been postulated for proteins involved in cargo selection or in assisting vesicle formation and fission at the TGN (De Matteis & Godi, 2004). Proteins with a function in endosomal trafficking—such as Hrs, early endosome antigen 1 (EEA1) or the late endosomal PIK-fyve (Fig 3)—recognize PI(3)P, most often through FYVE domains (Lindmo & Stenmark, 2006).

Given the plethora of PI-dependent functions, a crucial question is how the same PI species can regulate different cell physiological processes within the same membrane, for example, PI(4,5)P_2 at the

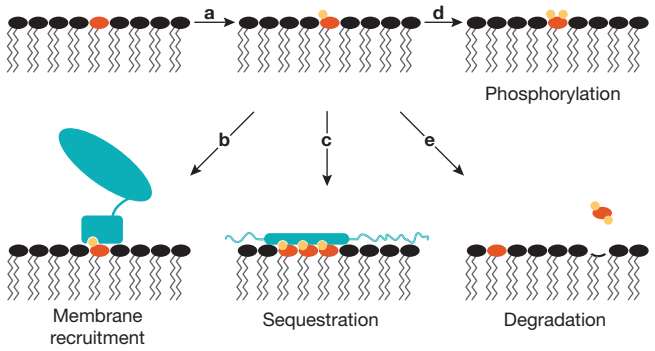

Fig 2 | Possible mechanisms for synthesizing and maintaining spatially restricted phosphoinositide pools. Spatiotemporal regulation of PI pools, for example, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) as schematically depicted here, might be achieved by a combination of several mechanisms, including locally restricted PI synthesis by regulated consecutive activation of PI kinases (a, d), recruitment of PI-specific effector proteins (b) or protein-mediated sequestration (c), and protection of the PI headgroup from enzymatic hydrolysis and degradation (e). PI, phosphoinositide.

plasma membrane. Although selective recruitment and activation of distinct PI kinase isozymes might contribute to this (Fig 2A), additional mechanisms are likely to operate. These might include sequestration of PIs by PI-binding effectors (as discussed above; Fig 2B) as well as by factors that can act as selective and reversible PI sinks (Fig 2C). Myristoylated alanine-rich C-kinase substrate (MARCKS) and related proteins contain stretches of basic amino acids that can bind up to three molecules of $PI(4,5)P_2$. They are highly abundant—at a concentration of 1–10 μM—and theoretically could buffer all $PI(4,5)P_2$ present in the plasma membrane. The association of MARCKS with the membrane surface is reversibly controlled by protein kinase C-mediated phosphorylation and binding to the calcium-binding protein calmodulin (CaM), which provides a mechanism to release the pool of pre-formed $PI(4,5)P_2$ following the appropriate signal (McLaughlin & Murray, 2005). Similar to MARCKS, clusters of basic amino acids are found in cytoplasmic regions of ion channels, such as inwardly rectifying and voltage-gated potassium channels, voltage-gated calcium channels and signalling receptors, including EGFRs (McLaughlin & Murray, 2005). Proteins with an equivalent function on intracellular membranes have not yet been identified.

Some $PI(4,5)P_2$-binding proteins, such as GAP-43, display a selective association with cholesterol-enriched membrane microdomains either owing to their association with cytoskeletal proteins or through partitioning into glycosphingolipid/cholesterol-enriched rafts. Sequestration of $PI(4,5)P_2$ into rafts has been speculated to contribute to the generation and maintenance of locally restricted PI pools (Golub & Caroni, 2005).

Different fates of PIs

The phosphorylation of $PI(4,5)P_2$ to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) by PI(3)K in response to growth-factor stimulation is well established (Fig 2D), and has been extensively described elsewhere (Lindmo & Stenmark, 2006, and references therein). Alternatively, $PI(4,5)P_2$-dependent signalling and trafficking processes can be regulated by phospholipase-mediated cleavage (Berridge & Irvine, 1989; Martin, 1998) or attenuated by removal of individual phosphate residues (Fig 2E). Several plasma membrane-targeted lipid phosphatases have been identified so far, including the polyphosphoinositide phosphatase synaptojanin, phosphatase and tensin homologue (PTEN), SH2-containing inositol phosphatase (SHIP) and a recently identified group of 4-phosphatases (Di Paolo & De Camilli, 2006). Synaptojanin is concentrated at nerve terminals, where it has a role in the uncoating of endocytosed synaptic vesicles. Functional studies have revealed several modes of regulation: synaptojanin is targeted to the plasma membrane through its proline-rich domain (PRD)-mediated association with endophilin (Fig 3), which also stimulates its 5-phosphatase activity. In addition, lipid phosphatase activity is regulated by cycles of phosphorylation and dephosphorylation (Wenk & De Camilli, 2004). Under resting conditions, most synaptojanin is phosphorylated due to the activity of cyclin-dependent kinase 5 (Cdk5); however, on stimulation, it becomes dephosphorylated by the calcium-dependent protein phosphatase calcineurin. Phosphorylation by Cdk5 or tyrosine kinases, like ephrinB (EphB) receptors (Irie *et al*, 2005), reduces its affinity for endophilin and strongly attenuates phosphatase activity. Therefore, at the synapse, both enzymes—PIP(5)K Iγ and synaptojanin—are simultaneously activated by calcium influx (Wenk & De Camilli, 2004).

Rab5 triggers the generation of PI(3)P in the endocytic pathway through the stimulation of Vps34, and the generation of an enzymatic cascade resulting in the activation of PI 5-phosphatases and PI 4-phosphatases during endosomal progression (Shin *et al*, 2005). Oculocerebrorenal syndrome of Lowe (OCRL), which is another 5-phosphatase, has been implicated in membrane traffic at the TGN–endosomal boundary, where it associates with clathrin-coated microdomains.

Notably, the inositol phosphatases OCRL and synaptojanin contain peptide motifs within natively unfolded regions that target enzymatic activities to clathrin- and adaptor-containing membrane sites (Fig 3). These include AP-2-binding WxxF, DxF and FxDxF motifs, eps15 homology domain ligands (NPF) and clathrin-interaction sites. In the case of synaptojanin 1, differential splicing of its motif domain contributes to the spatiotemporal control of its association with clathrin-coated pits (Perera *et al*, 2006). Whether and how such clathrin- and adaptor-binding motifs also regulate enzymatic activities of synaptojanin or OCRL remain to be determined.

The physiological importance of regulated PI degradation is also underscored by the association of PI phosphatases with genetic diseases (Pendaries *et al*, 2003), including bipolar disorder, Down syndrome (synaptojanin), cancer (PTEN) and Lowe syndrome (OCRL; Table 1).

Signalling cascades affected by membrane trafficking

Changes in the balance between receptor retrieval from and delivery to the plasma membrane effectively modulate the strength and duration of a cell's response to a given extracellular stimulus. However, some receptors resume signalling after their internalization and, in these cases, the final output of the signalling process might differ notably from that elicited at the plasma membrane. Therefore, subtle changes that retard or accelerate endosomal progression of a stimulated receptor can notably alter the pattern of elicited downstream-signalling cascades (Polo & Di Fiore, 2006). For example, signalling of activated EGFRs is mediated by mammalian son-of-sevenless (mSOS), Src-homologous and collagen-like substrate (Shc) and

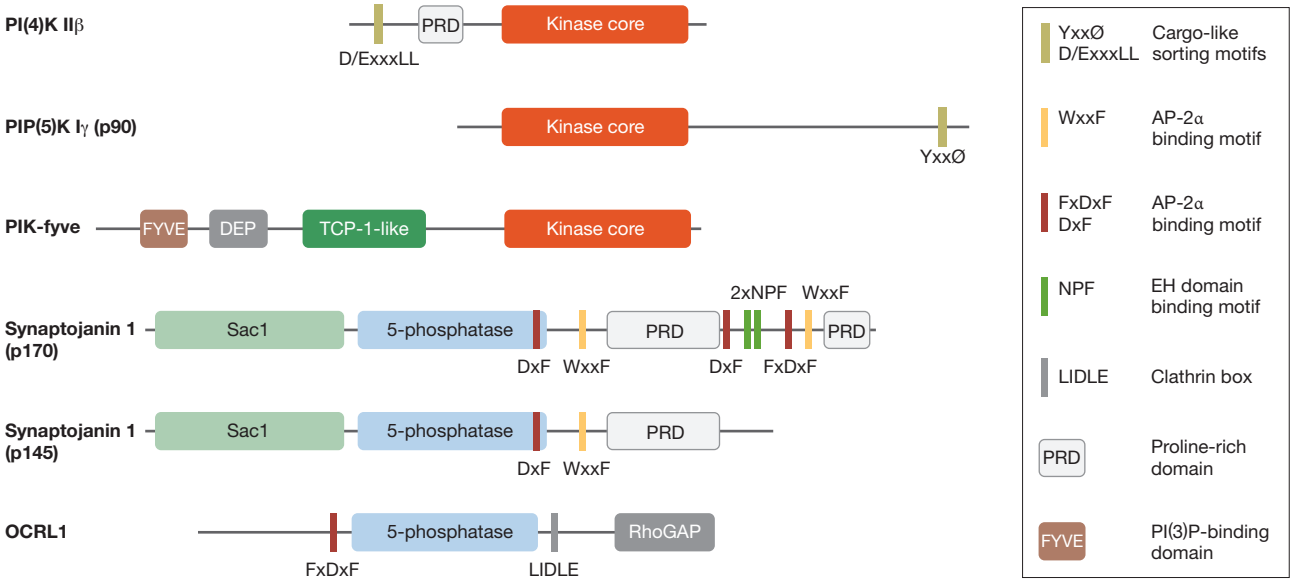

Fig 3 | Domain structure of major phosphoinositide-metabolizing enzymes. PI phosphatases, such as synaptojanin 1 and oculocerebrorenal syndrome of Lowe (OCRL), harbour clathrin and adaptor protein complex 2 (AP-2) adaptor-binding peptide motifs that target the enzymatic activity to clathrin-coated pits. Rho-GTPase activating protein (RhoGAP) or proline-rich domains (PRDs) might aid in targeting OCRL or synaptojanin 1 to specific intracellular or actin-rich membrane sites. Targeting domains or peptide motifs within PI kinases remain less well defined, although some enzymes, such as PIP(5)K Iγ-p90 or PI(4)K IIβ, contain cargo-like sorting motifs that might associate with AP adaptor complexes. PIK-fyve harbours a phosphatidylinositol 3-phosphate (PI(3)P)-specific binding FYVE domain aiding localization to the endosomal-lysosomal system. DEP, domain present in Disheveled, EGL-10 and Pleckstrin; PI, phosphoinositide; PI(4)K, phosphatidylinositol 4-kinase; PIP(5)K, phosphatidylinositol phosphate 5-kinase; Sac1, yeast suppressor of actin 1; TCP-1, tailless complex polypeptide-1.

EGF-receptor pathway substrate-8 (Eps8) in early endosomes, and by p14-MP1 in late endosomes. Transforming growth factor-β (TGF-β) receptors remain associated with Smad anchor for receptor activation (SARA) and Smad2 on early endosomes. Owing to the presence of a FYVE domain, signalling through SARA is restricted to Rab5-positive microdomains enriched in PI(3P), whereas it is excluded from Rab4-positive or Rab11-positive areas (Miaczynska *et al*, 2004).

Open questions and future directions

In the past few years, the role of PIs in cellular signalling and trafficking processes has been firmly established. However, we still lack information about the precise spatial and temporal regulation of distinct PI-modifying enzymes, and the lipid species they produce, especially at the single-cell level. Although the idea that PIs are localized to distinct subdomains or pools within the same compartment is attractive, at this point it seems difficult to prove experimentally. Imaging of PI turnover has been greatly advanced by the use of green fluorescent protein-tagged protein domains that specifically recognize distinct PI species with different subcellular localizations; however, these tools have limitations. Some PI-binding domains interfere with regular cellular functions, either by lipid sequestration or by competing with endogenous PI-binding proteins, whereas others provide additional binding sites for other cellular proteins. One possibility to circumvent these problems could be the application of PI-selective probes that undergo Förster resonance-energy transfer (FRET) only on binding to specific membrane lipids. Fluorescent probes with specificity for non-PI lipids, such as phosphatidic acid, are also lacking.

Some lipid-modifying enzymes are recruited and activated by the same factors, but have opposing effects with regard to their activities, for example, the 4-phosphatase and 5-phosphatase synaptojanin and type I PIP(5)K. How enzyme functions are coordinated to avoid a waste of energy remains an open question. Studies on the role of some lipid-modifying enzymes have been further complicated by the presence of different isozymes, including alternatively spliced variants, which mediate the same enzymatic reaction but are targeted to distinct subcellular domains. Therefore, genetic tools paired with sensitive mass-spectrometric analytical methods, targeted acute depletion of PI pools (Suh *et al*, 2006; Varnai *et al*, 2006) and the development of new specific pharmacological inhibitors might become important tools for tracking down the roles of distinct pools of PIs and PI-metabolizing enzymes.

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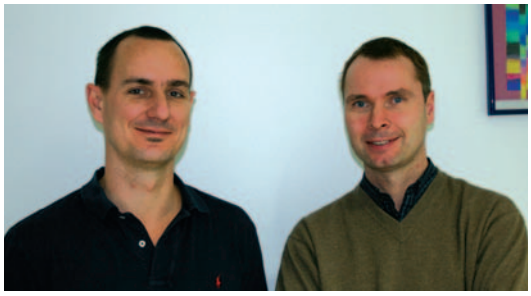

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